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Review

Antigene, Ribozyme and Aptamer Nucleic Acid Drugs: Progress and Prospects

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Nucleic acids are increasingly being considered for therapeutic uses, either to interfere with the function of specific nucleic acids or to bind specific proteins. Three types of nucleic acid drugs are discussed in this review: aptamers, compounds which bind specific proteins; triplex forming (antigene) compounds; which bind double stranded DNA; and ribozymes (catalytic RNA), which bind and cleave RNA targets. The binding of aptamers to protein may involve specific sequence recognition, although this is not always the case. The interaction of triplex forming oligonucleotides or ribozymes with their targets always involves specific sequence recognition and hybridization. Early optimism concerning the possibility of designing drugs without *a priori* knowledge of the structure of the target (except a nucleotide sequence) has been tempered by the finding that target structure has a dramatic effect upon the hybridization potential of the nucleic acid drug. Other obstacles to the creation of effective nucleic acid drugs are their relative high molecular weight (>3300) and their sensitivity to degradation. The molecular weight of these compounds has created a significant delivery problem which needs to be solved if nucleic acid drugs are to become effective therapies.

KEY WORDS: aptamers; antigene; drug delivery; triple helix; oligonucleotides; catalytic RNA; ribozyme.

INTRODUCTION

The central dogma of genetics states that information within cells flows from a repository molecule (DNA) to functional molecules (proteins) via a specific template molecule (mRNA). Transcription of RNA from DNA and translation of protein from RNA are each regulated carefully, since managing information flow is vital to the proper functioning of the cell. Each conversion permits amplification of the information—from a single gene, multiple RNA transcripts are produced, from which even more protein molecules are translated. Generalized interference with transcription and translation would obviously be detrimental to the cell. Modulation of specific gene expression and noting concomitant changes in cell physiology and function can provide critical insight into the biological function of particular genes. Specific modulation of gene expression may also be beneficial in inflammatory, neoplastic or viral disease since it would be desirable to affect only the inflammatory genes, oncogenes or viral genes without harming the normal functions of the cell (1).

The majority of currently employed pharmacologic approaches to modulation of gene function rely upon the interaction of low molecular weight chemical compounds with protein targets so as to alter the function of the protein. The past fifteen years have seen the embryogenesis of new therapeutic strategies, in which nucleic acid polymers are used to alter protein function or to interfere with the function of nucleic acids encoding the target protein. Nucleic acid drugs can be subdivided into four classes based upon their target site and mechanism of action (Table I): aptamers, antigene compounds, catalytic or ribozyme nucleic acids, and antisense compounds.

The intent of this article is to introduce the pharmaceutical scientist to aptamers, antigene compounds and ribozymes, and to review reports of biological effects produced by these informational drugs. The emphasis is placed upon biological effects observed in cultured cells, particularly when the gene target is of therapeutic interest, although *in vitro* studies are mentioned when significant. Antisense oligonucleotides or antisense RNA strategies will not be discussed—the reader is instead referred to several excellent reviews concerning the chemistry and biology of these nucleic acid drugs (3-6).

APTAMERS

Aptamers, from the Latin *aptus*, to fit, are single-stranded or double-stranded nucleic acids which are capable of binding proteins or other small molecules (7). Aptamers as therapeutics would most likely bind proteins involved in the regulation and expression of genes (*i.e.* transcription factors). The presence of the aptamer would act as a sink for the protein factors, preventing the factors from carrying out their normal functions and presumably modulating the ex-

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Table I. Classes of Nucleic Acid Drugs

	APTAMER	ANTIGENE	CATALYTIC	ANTISENSE
target	protein	duplex DNA	mRNA	mRNA
drug type	DNA/RNA	DNA/RNA	RNA	DNA/RNA
mechanism	binding: interfere with biological function	triplex formation: block transcription	hybrid assembly: cleavage and destruction of target	hybrid assembly: translation arrest and/or RNase H activation
site of action	intra- and/or extracellular	nucleus	nucleus and/or cytoplasm	nucleus and/or cytoplasm

pression of the genes dependent upon the activity of the protein.

To date, only a few instances of oligonucleotide aptamers displaying biological effects have been reported (Table II). Double-stranded phosphorothioate oligonucleotides capable of binding either octamer of NF-κB transcription factors were used to specifically modulate expression of a transfected CAT reporter gene or the endogenous IL-2 gene (PHA-stimulated) in B-cell and T-cell lines (8). Single-stranded oligonucleotides, when used at the same concentrations (7.5 μM) had no effect upon target gene expression in either case, suggesting the oligonucleotide was not behaving as an antisense or antigene compound. Transfected reporter genes lacking the consensus binding sequences for the octamer or NF-κB factors were also unaffected by the double stranded phosphorothioates. A similar approach was utilized to modulate CAT expression in MOLT-4 T-cells, although an aptamer effect versus other modes of inhibition was not conclusively demonstrated (9).

In addition to targeting proteins which are involved in regulating gene expression, aptamers may also bind proteins that perform other regulatory functions. Using an *in vitro* amplification and screening process, Bock and coworkers isolated a 15-mer DNA aptamer that bound human thrombin, a protein which previously had no known specificity for nucleic acids (10). The aptamer could prevent thrombin-catalyzed coagulation *in vitro* at nanomolar concentrations. Anticoagulant effects were also exhibited by this aptamer *in vivo* (11). The thrombin aptamer displays a novel G-quadriplex like structure (12,13) and appears to produce its biological effects by competing with fibrinogen binding at the anion-binding exosite (14; see Figure 1). A significant advantage of this approach is that the target is extracellular, so the aptamer nucleic acid does not have to cross cell membranes after parenteral administration.

A second aptamer approach has been reported (15,16). This "RNA decoy" strategy involves intracellular production of RNA transcripts containing defined secondary structures (TAR elements) known to interact with the HIV-1 *tat* protein. These decoys then compete with the viral RNA TAR element for *tat* binding, and thereby prevent HIV-1 replication by sequestering the *tat* protein. RNA decoys expressed were either chimeric $tRNA^{Met}$-TAR transcripts (allowing high transcript number via RNA polymerase III driven expression; 15), or HIV-1 LTR driven transcripts containing up to 50 copies of the TAR element per transcript (16).

ANTIGENE NUCLEIC ACID COMPOUNDS

Nucleic acids targeted to genomic DNA have been termed *antigene* nucleic acids (4). Antigene nucleic acids are designed to bind to single-stranded or double-stranded DNA. Binding of single-stranded DNA may occur at a replication or transcription bubble, and thereby interfere with either of these processes, although gene specificity would be expected only when the antigene compound interferes with transcription processes (see 17 for an example of selective inhibition of DNA replication). Alternatively, the antigene compound may bind the major groove of double-stranded DNA and form a triple helix (or *triplex;* Figure 2). Triplex formation may then prevent the interaction of various protein factors required for transcription, or it may physically block the initiation or elongation of the transcription complex.

Triplex Sequence Motifs. Like Watson-Crick base pair-

Table II. Aptamer Studies

Target species or inhibition goal	Target protein or DNA element	Cell line	Aptamer	Reference
transfected CAT, PHA-induced IL-2 expression	NF-κB, octamer transcription factors	B-cells, T-cells	double stranded phosphorothioate oligonucleotide	(8)
transfected CAT	adenosine deaminase enhancer	T-cells	double stranded phosphorothioate oligonucleotide	(9)
anti-coagulation	human thrombin	*in vitro, in vivo* assays	single stranded phosphodiester oligonucleotides	(10, 11)
inhibition of HIV-1 expression	HIV-1 *tat* protein	human T cell lines; COS cells	RNA transcripts from expression vectors	(15, 16)

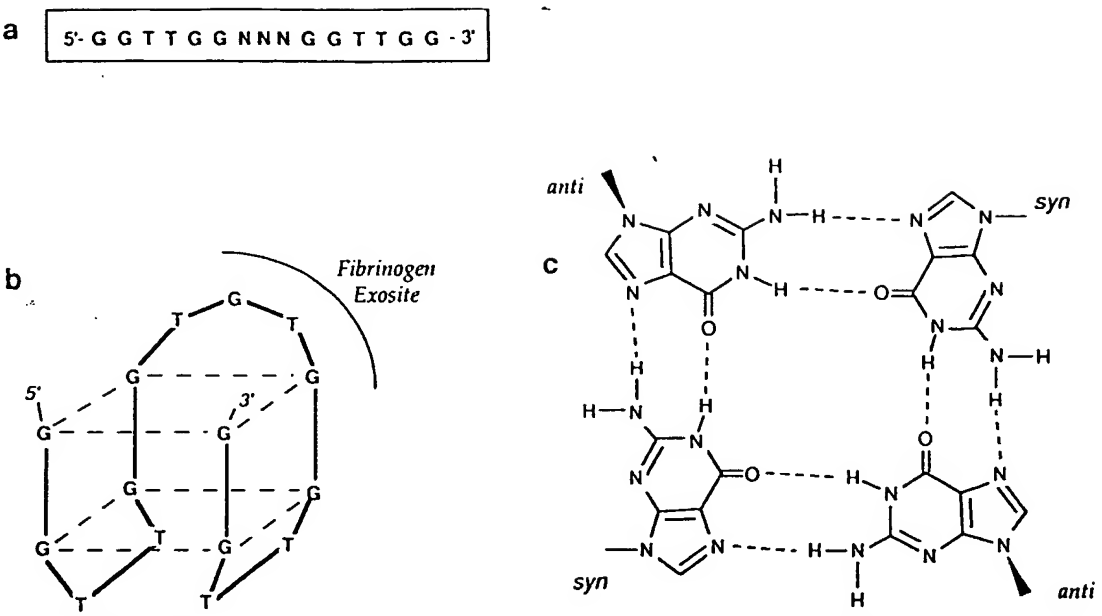

Figure 1. Sequence and structure of the thrombin binding aptamer. a) The consensus sequence of the thrombin aptamer as determined from an *in vitro* selection procedure (10). b) Schematic tertiary structure of the thrombin aptamer as deduced from NMR-studies and X-ray crystallography of the aptamer-thrombin complex (12-14). The bold lines represent the aptamer backbone, while the dashed lines denote the G-quartets. Note that in the crystal structure of the thrombin-aptamer complex, the guanosines in the top quartet are coplanar, while the bottom quartet is not completely coplanar. Not shown is the potassium ion located roughly at the center of the cube. c) Arrangement of the four guanosines in a G-quartet, in alternating *syn* and *anti*-conformations.

ing, the molecular recognition processes behind triplex formation are quite specific and inherently due to hydrogen bond donor and acceptor moieties on the purine and pyrimidine bases of the nucleotides (Figure 2). One of the best understood triplex binding motifs involves binding of homopyrimidine oligonucleotides to the major groove of duplex DNA at homopurine-homopyrimidine sequences. Homopyrimidine oligonucleotides bind parallel to the duplex homopurine strand via formation of C^+-G-C and T-A-T triplets (18). Triple helices can also be formed at homopurine-homopyrimidine duplex DNA sites by antiparallel binding of a homopurine oligonucleotide (19). Additionally, oligonucleotides containing G and T residues will form triplexes with DNA target sites (20-23). The orientation of the binding of the G and T triplex-forming oligonucleotides is dependent upon the exact sequence of the target site (24). α-oligonucleotides have been shown to form triple helices at homopurine-homopyrimidine duplex DNA target sites (25). Some triplex-forming oligonucleotides have been designed to "switch" strands when blocks of homopurine sequences alternate between strands at the duplex DNA target site (19, 26, 27). Based upon the studies of triplex-forming oligonucleotides performed to date, it appears that in the absence of chemical modification, a triple helix forming sequence should contain at least 20 bases in order to bind its target site with sufficient affinity so as to achieve biochemical effects (see Table III).

Although antigene compounds target double stranded DNA, triplex formation may occur at appropriate sites on other nucleic acids. RNase H directed cleavage of an RNA-DNA heteroduplex has been shown to be inhibited *in vitro* by a DNA triplex forming oligonucleotide, a result which makes possible another approach for anti-HIV nucleic acid therapeutics (28). Triplex formation also has been demonstrated using an oligonucleotide that bound a helical region of a single-stranded nucleic acid (29), or using bipartite oligonucleotide "clamps" which first bind single stranded nucleic acids to form a duplex, followed by folding over of the oligonucleotide to allow triplex formation (30).

It is obvious that triplex formation by homopyrimidine oligonucleotides requires an acidic pH, since C^+-G-C triplet formation is predicated upon protonation of the cytosine. Several modified bases have been substituted for cytosine in order to extend formation of homopyrimidine oligonucleotide-directed triple helices to the physiological pH range. These bases include 5-methyl cytosine (31), methyl-pseudocytidine (32), N^6-methyl-8-oxo-2'-deoxyadenosine (33), 3-methyl-5-amino-1H-pyrazole[4,3-d]pyrimidin-7-one (34), 7,8-dihydro-8-oxoadenine (35) and 1-propynyl cytosine (36).

The kinetics of triplex formation has been shown to be slower than the kinetics of duplex formation (37, 38) where the rate-limiting step appears to be the nucleation of 3 to 5 base-triplets (38). Due to this 3 to 5 base-triplet requirement, triplex formation is very sensitive to mismatches, demonstrating that triplex formation should display the required selectivity necessary for specific gene regulation (39). Dissociation of the triplex also appears to be slower than duplex dissociation, with one triplex-forming oligonucleotide displaying a half-life of approximately 12 hours (37).

Intercalating agents or cholesterol groups covalently attached to the triplex-forming oligonucleotides increase the stability of the triplex (Figure 2; 25, 40-42). Alternatively, triplex stabilization can be achieved using aromatic com-

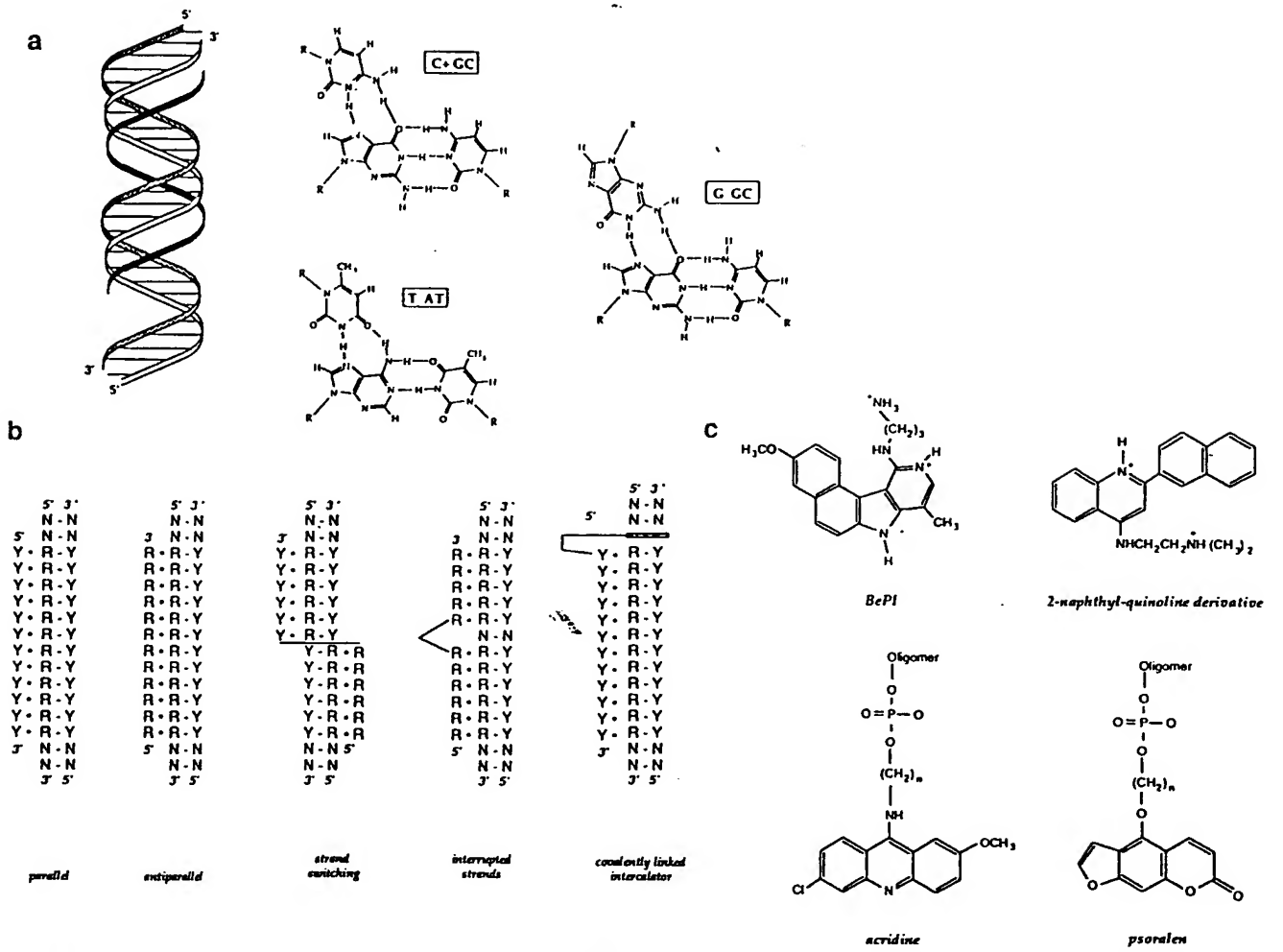

Figure 2. Structures important in oligonucleotide-directed triplex formation. a) Schematic of an oligonucleotide bound to a duplex to form a triple helical structure, and the arrangement of bases in C$^+$GC, TAT, and GGC triplets. b) Possible binding modes for triples oligonucleotides. Shown are parallel and antiparallel binding modes for homopurine-homopyrimidine target regions (18,19), "strand-switching" oligonucleotides (26,27), binding to interrupted homopurine-homopyrimidine target regions by oligonucleotides containing an abasic linker designed to span the interrupting duplex nucleotides (194), and an oligonucleotide conjugated to an intercalating agent to increase the binding affinity (40-42). R = purine (A or G); Y = pyrimidine (C or U). c) Structures of compounds used to increase oligonucleotide affinity or activity at the triplex site. Several polyaromatic compounds have been synthesized which preferentially bind and stabilize triple helices, including a benzo[e]pyridoindole derivative (BePI; 43, 44) and a 2-naphthyl quinoline derivative (45). Acridine derivatives have been covalently linked to the 5' termini of triplex forming oligonucleotides in order to improve the affinity of the oligonucleotide for the triplex site (40). Psoralen derivatives have also been covalently linked to triplex forming oligonucleotides to afford sequence-specific DNA cross-linking reagents (48)

pounds which intercalate into triple helices (43-45). If the aromatic compound is covalently linked to the oligonucleotide, linker length and composition may strongly influence the enhancement of oligomer binding due to the intercalator (46). Covalently bound triplexes have been produced using chemically-activated alkylating bases incorporated into the oligonucleotide (47) or by photo-activated cross-linkers attached to the triplex forming oligonucleotide (48, 49). Finally, triplex-forming oligonucleotides have been conjugated to chemically activated (18, 50, 51) or photo-activated (52) cleavage reagents, affording creation of "artificial endonucleases" capable of cleaving duplex DNA.

To date, the majority of antigene compounds reported have been DNA oligonucleotides or their analogs. Skoog and Maher investigated the use of both DNA and RNA oligonucleotides as antigene compounds in a cell-free system utilizing the bacteriophage T7 RNA polymerase promoter (53). As expected from previous studies (54), both homopurine and homopyrimidine DNA oligonucleotides were capable of repressing the T7 promoter. However, only the homopyrimidine RNA oligonucleotides could bind duplex DNA. Homopurine RNA oligonucleotides did not form triplexes, unless partially substituted with deoxyribose residues.

Biochemical Effects of Antigene Compounds Observed in Vitro. Formation of triple helices *in vitro* has been shown to inhibit the binding of restriction endonucleases and restriction methylases (55-57), as well as transcription factors (56,58) if the triplex site spans or overlaps the sequence recognized by the protein. Oligonucleotide-directed triplex formation also has been shown to inhibit transcription processes *in vitro*, including bacteriophage T7 transcription initiation (54, 59, 60), T7 and T3 transcription elongation (61),

Table III. Antigene Nucleic Acid Studies Within Eukaryotic Cells

Target gene	Cell line	Oligomer size, modifications	Reference
Transfected genes			
CAT gene/IL-2Rα promoter	HSB2 cells (T-cell)	15-mer, acridine or psoralen linked	(49, 68)
CAT gene/(6–16) IRE	HeLa cells	21-mer	(69)
CAT gene/*tk* promoter PRE upstream	CV-1 cells	38-mer, cholesterol	(70)
Endogenous genes			
IL-2Rα	human lymphocytes	28-mer	(22)
c-myc	HeLa cells	27-mer	(71, 72)
SV40 T Ag	tsa 8 cells	15, 20-mer PNAs	(66)
Antivirals			
SV40	CV-1	8-mer, acridine	(17)
HIV-1	MT_4 U937/HIV-1	31, 38-mers	(23)

E. coli polymerase elongation (62), and human RNA polymerase II elongation (47) or Klenow fragment action *in vitro* (63). Often the triplex block resulted in the production of truncated transcripts. However, stalled polymerases eventually read-through the triplex block (47). Covalent crosslinking of oligonucleotides to duplex DNA increases the amount of truncated transcripts produced (47) or may block transcription initiation (64), although this block may be reversible *in vivo* due to DNA repair enzymes (65).

Peptide nucleic acids (oligomers with peptide-like backbones instead of phosphodiester backbones, PNAs; (67)) have been also shown to block human RNA pol II elongation *in vitro*, as well as prevent the action of restriction endonucleases upon double stranded DNA (66). However, the mechanism of inhibition by PNA is likely not triplex formation, but rather strand invasion by the PNA into the DNA duplex at AT rich sites (66, 67).

Results Obtained Using Antigene Compounds in Cells. Currently, a handful of reports have appeared in which oligonucleotides have been used as antigene compounds within cells. These studies are summarized in Table III, where they are divided into classes based upon the type of gene targeted. Most studies first demonstrate triplex formation in solution, and then infer the biological effects observed in cell culture systems in the presence of the triplex forming oligonucleotide are due to triplex formation.

The NF-κB transcription factor binding site located near a purine rich sequence of the interleukin-2 receptor subunit α (IL-2Rα) promoter has been targeted in several studies of antigene compounds. Grigoriev and coworkers demonstrated triplex formation and inhibition of NF-κB transcription factor binding by an acridine-derivatized 15-mer homopyrimidine oligonucleotide targeted to this site (68). Transfection of an IL-2Rα/CAT reporter construct, preincubated with 5-10 μM oligonucleotide, into a human T-cell line demonstrated specific dose-dependent inhibition of CAT expression. CAT expression from a control construct, or from a mutant reporter construct lacking the NF-κB and oligonucleotide binding sites, was unaffected by the oligonucleotide. Further cellular studies utilized a psoralen conjugated oligonucleotide which allowed cross-linking to this triplex site *in vitro* or within transfected cells (49). Orson and colleagues utilized a 28-mer oligonucleotide targeted to this region and demonstrated triplex formation *in vitro* (22). When the oligonucleotide was administered to human peripheral blood T-lymphocytes for 2 hours prior to PHA-stimulation, specific reductions in IL-2Rα mRNA were observed while *c-myc*, β-actin, IL-6 and Il-2Rβ mRNA levels were unchanged. Maximal suppression of IL2Rα mRNA was 32% relative to control, using 20 μM oligonucleotide. Nuclear run-on experiments also demonstrated reductions in the rate of IL2Rα transcription relative to *c-myc* and β-actin transcription. However, no direct evidence was presented for triplex formation in the cells.

Roy designed several oligonucleotides to bind the purine-rich region of the interferon response element (IRE) from the 6-16 gene (69). Co-transfection of HeLa cells with a 6-16/IRE-CAT construct and a 21-base triplex forming oligonucleotide prevented interferon-induced CAT expression. The inhibition was dependent upon the oligonucleotide dose, with half-maximal inhibition occurring at 50-100 nM oligonucleotide.

Ing and coworkers reported that 38-mer oligonucleotide could form a triplex within the progesterone response element (PRE) *in vitro* (K_d ~ 100 nM), block binding of progesterone receptors to their target site *in vitro*, and inhibit *in vitro* transcription of reporter genes responsive to progesterone stimulation (70). The 38-mer was also derivatized with cholesterol and administered in culture medium to transfected CV-1 cells, where it inhibited transcription of PRE-containing CAT reporter gene constructs (50% inhibition ~ 20 μM oligonucleotide).

Several groups have targeted the *c-myc* proto-oncogene in HeLa cells. Hogan's group used a 27-base long purine-rich DNA oligonucleotide targeted 115 base pairs upstream of the *c-myc* P1 promoter to repress *c-myc* transcription in HeLa cell extracts (20). Nuclei isolated from oligonucleotide-treated HeLa cells (125 μM) showed DNase-mediated cleavage to be inhibited at the triplex target site, suggesting triple helix formation was occurring within the cells (71). Steady-state levels of mRNA from the *c-myc* P1 promoter were reduced relative to those from the *c-myc* P2 promoter and relative to β-actin mRNA levels, suggesting the oligonucleotide was specifically affecting P1-driven *c-myc* transcription. The midpoint of oligonucleotide-mediated inhibition of P1 transcripts was approximately 25 μM, whereas the K_d mea-

sured *in vitro* was approximately 500 nM (20). This same oligonucleotide was tested in HeLa and SKOV-3 cells when covalently linked to acridine and was able inhibit the proliferation of these cells (72).

As mentioned above, PNA oligonucleotides display strong suppression of transcription *in vitro*, although suppression may be due to strand-invasion (leading to very stable PNA-nucleic acid duplexes) rather than triplex formation. Two 15-mer and 20-mer PNA oligonucleotide were targeted to the SV-40 large T antigen (T Ag) gene, and microinjected into tsa 8 cells, a cell line containing a single-copy integrant of the SV 40 early region. At intracellular concentrations of ~1 μM, significant inhibitions of T Ag expression were noted without reductions in the expression of a co-injected β-galactosidase expression construct (66). The authors suggest that this inhibition is likely due to an antisense mechanism, but cannot rule out an antigene mechanism.

Antiviral Antigene Targets. Birg and coworkers describe the antiviral effects of an octathymidylate-intercalator conjugate tested in CV-1 cells infected with SV 40 (17). The derivatized octathymidylate specifically inhibited the cytopathic effects of SV 40 in CV-1 cells up to 95% (at 30 μM), whereas non-sequence specific acridine-linked oligonucleotides and underivatized octathymidylates had no inhibitory effects. The authors discuss possible modes of viral inhibition, but favor the interpretation that either duplex or triplex formation leads to inhibition of viral DNA replication.

McShan and coworkers reported HIV-1 inhibition by two different mixed purine-pyrimidine oligonucleotides targeted to the transcription initiation site and the nuclear factor Sp1 binding site of the HIV-1 genome (23). These 31-base and 38-base oligonucleotides were blocked at their 3′ termini with amine groups, which conferred resistance to cellular and serum nucleases. Studies of the antigene oligonucleotides showed micromolar association constants. Both triplex forming oligonucleotides added to cells at 10 μM showed substantial inhibition of HIV-1 associated cytopathology and p24 antigen production in MT_4 cells (acute HIV-1 model), while the more efficacious oligonucleotide displayed a dose-dependent inhibition of p24 antigen production in U937/HIV-1 cells (chronic HIV-1 model).

RIBOZYMES

Catalytic RNAs, or ribozymes, are RNAs which catalytically cleave covalent bonds in a target RNA (reviewed in 78, 79). The catalytic site is the result of the conformation adopted by the RNA-RNA complex in the presence of divalent cations. Catalytic activity requires a "ribozyme core" sequence and divalent cation. For most ribozymes, specificity is conferred by an internal guide sequence (a portion of the ribozyme RNA which base-pairs directly with the substrate RNA). RNase P, the endoribonuclease responsible for producing the 5′ ends of mature tRNA molecules (80) is a natural *trans*-acting ribozyme. RNase P, however, does not directly base-pair with its substrates; it is thought that the enzyme instead recognizes a particular structural domain in its substrate (81).

Cech and coworkers first described RNA self-splicing in their studies of the 413-nucleotide group I intron from *Tetrahymena thermophila* (82). Several years later, Zaug and colleagues described a variant of the *T. thermophila* ribozyme which could act in *trans* (i.e. as a sequence specific endoribonuclease acting upon other RNA substrates; 83). The work of Forster and Simons (84, 85) concerning plant virusoid RNAs suggested smaller RNA domains were capable of possessing ribozyme activity. Uhlenbeck was the first to demonstrate a small active ribozyme in which the catalytic and substrate stands were separated (86). Other simple ribozymes designed to act in *trans* were subsequently described (87, 88).

The first published comments concerning the potential use of ribozymes as therapeutic agents appeared shortly thereafter—Haseloff and Gerlach enumerated general design rules for simple hammerhead ribozymes capable of acting in *trans* - the requirement for a GUC containing target sequence, a conserved catalytic domain of defined sequence, and flanking helices which confer specificity to the ribozyme (89). In their study, three separate ribozymes were shown to cleave CAT RNA *in vitro* at three separate GUC target sites, depending upon the flanking sequences incorporated into the each ribozyme. The anti-CAT ribozymes also demonstrated true catalytic activity by participating in greater than 10 cleavage events in experiments involving a molar excess of substrate RNA. Subsequently, these ribozymes were tested in one of the earliest cellular studies of engineered ribozyme activity (90), where they were shown to inhibit CAT expression up to 60%.

Ribozyme Design Motifs. Although natural ribozymes can be divided into several broad classes based upon structure and mechanism (79), two structural classes are the basis for the design of most *trans* acting ribozymes (Figure 3): the *hammerhead* motif found in the self-cleaving domain of plus-strand satellite RNAs from tobacco ringspot virus (84) and the *hairpin* motif found in the self-cleaving domain of minus-strand satellite RNAs from tobacco ringspot virus (91-95).

A secondary structural model for hammerhead ribozymes appears in Figure 3. Extensive mutagenesis studies have been used to determine the minimal sequence conservation requirements for catalytic activity (96). Hammerhead motif ribozymes can be targeted to any NU(A/U/C)•N sequence, where the dot represents the site of cleavage (88).

The second main class of engineered, *trans*-acting ribozymes utilize the hairpin or paperclip motif. A simple model for the secondary structure of the hairpin ribozyme is shown in Figure 3 (97, 98). Hairpin ribozymes can be targeted to (A/G)(C/U)N•G(A/C/U)(C/U)(G/U/C) sequences (101), where the 2′-OH moiety on the substrate N ribonucleotide and the 2-amino group of the conserved guanosine are required (99, 208). The structural, sequence and ionic requirements of hairpin ribozyme itself have also been characterized (97, 100, 208-211).

RNase P contains a catalytic RNA subunit and a protein subunit (102). Forster and Altman have shown that *E. coli* RNase P can be directed to cleave RNAs which are not normally substrates for this enzyme, provided that target RNA hybridizes to an "external guide sequence" (EGS) RNA (103). The target RNA-EGS RNA complex adopts a conformation which mimics the structure of the natural pre-tRNA substrates, allowing cleavage *in vitro*. Human RNase

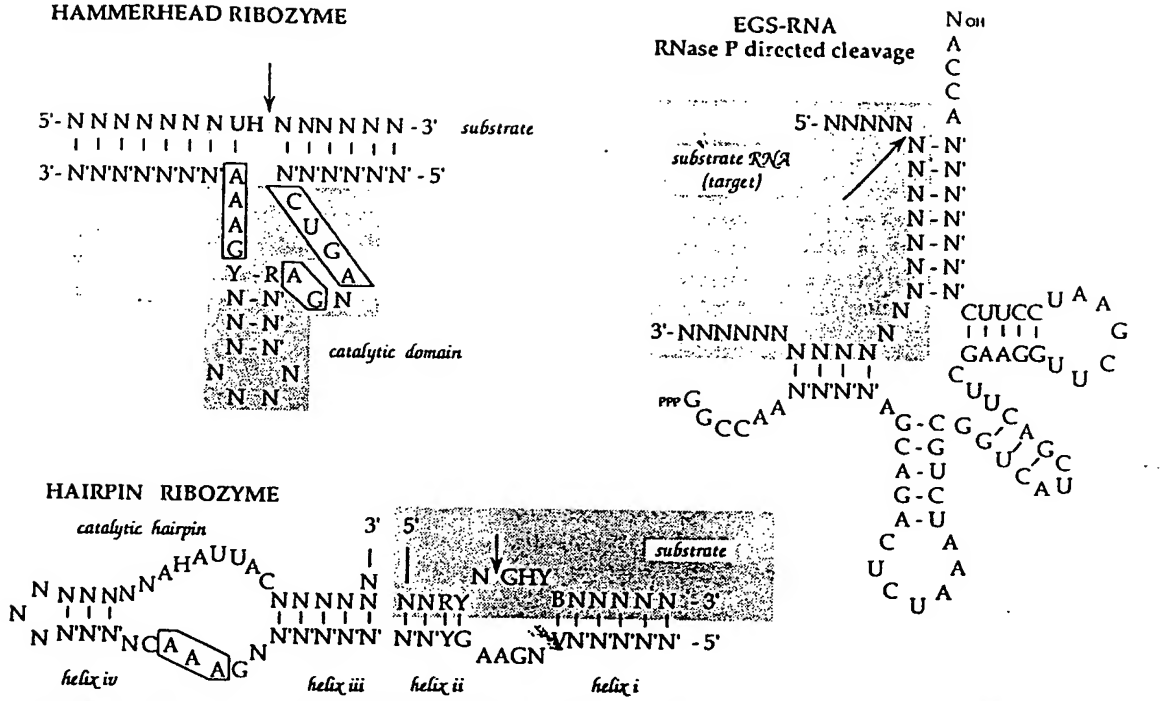

Figure 3. Secondary structural models of ribozyme-substrate complexes. *Hairpin ribozyme* (adapted from ref. 96). Hammerhead ribozymes recognize NUH sequences, cleaving after the H nucleotide (at arrow). The catalytic domain of the hammerhead is shaded, and the conserved nucleotides required for catalytic activity are indicated within the boxes. *Hairpin ribozyme* (adapted from ref 97). Hairpin ribozymes are named for the simple secondary structure model used to describe them. The substrate molecule donates the nucleotides shown within the shaded region; the substrate is cleaved 5' to the conserved G residue (at arrow). Helices I and II provide recognition and structural features required for catalysis; mutations in the "loop" region between helices III and IV produce catalytically inactive ribozymes which are still capable of substrate binding (97, 100). *EGS-RNA directed RNase P cleavage.* Human RNase P can be directed to cleave non-tRNA substrates *in vitro* provided a tRNA-like complex is formed by the target RNA molecule with an external guide sequence (EGS) RNA (103, 104). Shown above is a model complex (adapted from ref. 104). Single letter nucleotide codes are as follows: B = G, U, or C; H = A, C, or U; N = any of the bases, with N' = its Watson-Crick complement; R = A or G; V = A, C or G; Y = C or U (189).

P has also been directed to cleave CAT mRNA *in vitro* by use of an EGS-RNA, although the structure of the complex required for human RNase P recognition is more complicated than that of *E. coli* RNase P (Figure 3; 81, 104, 218).

The RNA self-cleaving activity associated with hepatitis delta virus (HDV) has been explored as a possible *trans*-acting ribozyme. To date, this ribozyme has not been engineered to act in *trans* upon non-HDV RNA substrates, although short oligonucleotide substrates have been targeted (105-109).

Ribozyme Kinetics. Ribozymes appear to obey simple Michaelis-Menten type kinetics (86, 110). As is the case for protein enzymes, the cleavage reaction catalyzed by ribozymes should, to some extent, be reversible. Hairpin ribozymes appear capable of catalyzing ligation reactions (111-113). Hammerhead ribozymes also have an intrinsic ligation capability, although the internal equilibrium of the ribozyme reaction favors cleavage product formation (110).

Under subsaturating conditions, cleavage rates are determined by the ribozyme k_{cat}/K_m. The most efficient wild-type ribozymes display k_{cat}/K_m values of 10^8 M^{-1} min^{-1} (about the order of the apparent hybridization rate constant for two oligonucleotide; 78). It has been suggested that within the intracellular environment, the hybridization step may be rate limiting for *trans*-acting ribozymes (114).

At substrate saturation, cleavage will be determined by the k_{cat} of the ribozyme, which in turn may reflect the cleavage step or the product release step. Kinetic and mutagenesis experiments have indicated that for both natural and engineered ribozymes, at substrate saturation, product release may be rate limiting (115-117). The theoretical underpinnings and implications of this observation are described by Herschlag (118). This release rate-limitation is due to the length of the internal guide sequence (base-paired) regions and the consequent effects upon nucleic acid dissociation kinetics. Additionally, it is expected that mismatched substrates will be cleaved if the substrate dissociation step is slow relative to the cleavage step (118, 119). Therefore studies have been carried out attempting to optimize the length of the hybridizing arms (e.g. 114, 120). Several reports have shown that certain non-specific RNA binding proteins can enhance the catalytic activity of ribozymes, most likely by facilitating both the binding and dissociation of the RNA strands (121, 122, 212). Oligonucleotide "facilitators" binding adjacent to the ribozyme recognition sites likewise improved the catalytic efficiency of a ribozyme (123).

Analysis of Ribozyme Activity. Demonstration of ribozyme activity *in vitro* is relatively straight forward—incubation of the ribozyme and its substrate, followed by polyacrylamide gel electrophoretic analysis, allows detec-

tion of the cleavage products. Studies performed in the test tube using engineered, *trans*-acting ribozymes are summarized in Table IV. Studying ribozyme activity in cells is slightly more complex. First, it requires a means of delivering the ribozyme to the cell. This is usually accomplished in one of three ways—delivery of pre-formed ribozymes by microinjection or by cationic liposomes; delivery by transfection with plasmid constructs designed to express the ribozyme RNA; or delivery by infection with a retroviral vector bearing the ribozyme. Second, cleavage products produced by the ribozyme usually cannot be detected within cells (two notable exceptions, 124, 125). The inability to detect cleavage products has been ascribed to the activity of intracellular nucleases which quickly degrade the cleaved substrate RNAs (90, 126). Therefore, indirect evidence is usually presented from which ribozyme activity in cells can be inferred.

Several investigators have used sets of PCR primers in a reverse transcriptase-PCR (RT-PCR) assay to detect changes in the ratios of full length to partial length RNA transcripts (127-129, 215). Other investigators have performed various control experiments which point to ribozyme activity. Since RNA transcripts designed to act as ribozymes may also act as antisense RNA molecules (124), the best controls from which ribozyme activity within cells can be inferred include use of simple antisense constructs identical to the internal guide sequence of the ribozyme but lacking a catalytic region, and/or use of ribozymes containing mutations in the catalytic region so as to render the ribozyme inactive. All of the studies of ribozyme activity within cells in which these controls have been performed demonstrate that changes in phenotype or reductions in target gene expression within ribozyme-transfected cells are, in part, due to antisense activity (124, 130-134).

Studies of Ribozyme Activity Within Cells. Cellular studies of engineered, *trans*-acting ribozymes are summarized in Table V, where they are divided into three broad groups, depending upon the target gene: ribozymes directed toward 1) coinjected/cotransfected substrates, 2) viral RNA targets, or 3) endogenous substrates.

Table IV. *Trans* Acting Ribozyme Studies (*in Vitro*) with Large RNA Target Molecules

Substrate RNA	Ribozyme type	Reference
potato leafroll viral RNA	hammerhead	(176)
HIV-1 *gag* and 5′ LTR	hammerhead	(177)
HIV-1 *vif* gene	hammerhead	(178)
HIV-1 LTR	hammerhead	(179)
HIV-1 R region	hammerhead	(180)
Hamster prion pre-mRNA	hammerhead	(181)
HBV RNA	hammerhead	(182)
Arenavirus RNAs (LCMV)	hammerhead	(183)
glutamine synthetase from *Phaseolus vulgaris*	hammerhead	(184)
Alzheimer amyloid peptide (βAPP)	hammerhead	(185)
murine calretinin mRNA	hammerhead	(120)
human multiple drug resistance gene (MDR-1)	hammerhead	(186)
bcr/abl transcript	hammerhead	(187) (169)
E. coli prolipoprotein signal peptidase	hairpin	(188)

Co-injection/transfection of Ribozyme and Substrate. Early studies of ribozyme activity in cells focused on ribozymes cotransfected or coinjected with their target substrate. The anti-CAT ribozymes designed by Haselhoff and Gerlach (89) were incorporated into luciferase expression constructs and co-transfected into COS-1 cells along with a CAT reporter construct (90). Ribozyme-mediated inhibition of CAT expression was noted (up to 60% inhibition versus controls) provided the ribozyme expressing transcript was in 1000-fold molar excess over the target RNA. Similarly, Cotten and Birnsteil (135) were able to show ribozyme-mediated suppression of U7 snRNA, but not a control RNA substrate, when both ribozyme and target RNAs were co-injected into *Xenopus* oocytes. Again, the suppression required a substantial molar excess (estimated to be 500 to 1000-fold) over the target RNA.

L'Huillier and coworkers studied several hammerhead ribozymes as well as a hairpin ribozyme targeted to α-lactalbumin in C127I cells (132). These investigators used a T7/vaccinia virus system to first deposit large amounts of T7 RNA polymerase in the cytoplasm of infected cells. The virally-infected cells were then transfected using the cationic lipid DOTAP(136) with DNA coding for both α-lactalbumin and the various ribozymes under the control of the T7 promoter. Northern analysis of the lipofected cells indicated up to 90% inhibition by some of these ribozymes. The authors observed that a hairpin ribozyme was slightly more effective than a hammerhead ribozyme targeted to the same site, and that in the *in vitro* activity of the ribozymes did not always correlate with the activity observed within the cells. A similar lack of correlation between *in vitro* and cellular ribozyme activities were noted in a study of anti-HIV ribozymes (137).

Altman and coworkers demonstrated that CAT mRNA could be cleaved by human RNase P *in vitro* when hybridized to an external guide sequence (EGS) RNA (104). Transfection of human lung cancer cells (A549) with both CAT RNA constructs and an external guide sequence RNA construct (which is required for RNase P directed cleavage, see above) resulted in decreased CAT expression and reduced levels of CAT RNA relative to controls (104). RNase P-mediated cleavage, however, could not be conclusively demonstrated since cleavage products were not detected.

Several groups have shown that hammerhead ribozymes directed to the *c*-Ha-*ras* gene can partly reverse the neoplastic phenotype when the ribozyme and activated oncogenes are cotransfected into NIH3T3 cells (138-140). Ribozymes in these studies were able to suppress foci formation, tumor cell growth, and the tumorogenicity of the cells when injected into mice.

Cellular Studies of Ribozymes: Antivirals. Ribozymes directed against viral RNA are the subject of several published reports. Xing and Whitton designed three different hammerhead ribozymes targeted against an lymphocytic choriomeningitis virus (LCMV), a member of the arenavirus family (141). The authors reported a diminution of viral RNA levels as well as reduced yield of infectious virus in the ribozyme-expressing cells challenged with LCMV, but pre-

Table V. Cellular Studies of Engineered, *trans*-acting Ribozymes with Potential Therapeutic Applications

Substrate RNA	Ribozyme motif	Expression via	Target cells	References
c-Ha-*ras*	hammerhead	plasmid	NIH3T3	(138–140)
			EJ cells;	(151)
			human melanoma	(152)
c-fos	hammerhead	plasmid	A2780S	(130, 131)
			A2780DDP	
α-lactalbumin	hammerhead	plasmid	C1271 cells	(132)
HIV-1 integrase	hammerhead	plasmid	*E. coli*	(163)
HIV-1 5′ leader	hammerhead	retroviral	MT_4	(142)
	hammerhead	preformed	SW480	(133)
HIV-1 5′ leader	hairpin	plasmid, retroviral	HeLa	(100, 145, 146)
HIV-1 U5	hammerhead	retroviral	H9, MT_4	(128)
HIV-1 *gag*	hammerhead	plasmid	$CD4^+$ HeLa	(127)
HIV-1 *env*	hammerhead	plasmid	$CD4^+$ HeLa	(144)
HIV-1 *tat*	hammerhead	retroviral	Jurkat T-cells	(143)
LCMV	hammerhead	plasmid	NIH3T3	(141)
Bovine Leukemia Virus	hammerhead	plasmid	bat lung cells	(129)
TNF-α	hammerhead	preformed	HL60, PBMNC	(154)
bcr/abl	hammerhead	preformed	EM-2 cells	(147–150)
		retroviral	K562 cells	
O^6-MGMT	hammerhead	plasmid	HeLa CCL2	(153)
MDR-1	hammerhead	plasmid	MOLT-3	(175)
			EPP85-181RDB	(213)
Pleiotrophin	hammerhead	plasmid	melanoma cells	(214)
Influenza A	hammerhead	plasmid	COS cells	(215)
β2-microglobulin	hammerhead	plasmid; transgenic mice	NIH/3T3	(216)
lck and *fyn* tyrosine kinases	hammerhead	retroviral	Jurkat T-cells	(217)

sented no evidence to distinguish ribozyme-mediated suppression from antisense-mediated suppression. Cantor and coworkers designed a ribozyme against the *rex* and *tax* transcripts of bovine leukemia virus (129). In bat lung cells expressing the ribozyme, PCR analysis suggested that cleavage was occurring. Substantial reductions in p24 (61%) and reverse transcriptase activity (92%) were also noted in these cells. Tang and colleagues characterized several COS cell subclones containing hairpin and hammerhead ribozymes targeted towards the viral segment 5 of influenza A virus (215). They observed up to 70-80% inhibition of viral superinfection in some of these subclones.

The majority of anti-viral ribozymes tested in cell systems have been directed at HIV-1. Hammerhead ribozymes have been directed to the *gag* transcript (127), the 5′ untranslated leader sequences or U5 region of the LTR (128, 133, 142), and the *tat* gene (143). Multitarget hammerhead ribozymes were constructed so that single RNA transcripts contained up to nine ribozymes targeted to different conserved regions of HIV-1 *env* RNA (144). Each of these studies demonstrated decreased or delayed viral production in ribozyme-containing cells relative to control cells, but evidence of ribozyme activity varied. In the studies of the anti-*gag* ribozyme (127), and anti-U5 ribozyme (128), an RT-PCR assay of full length to partial length HIV-1 RNA suggested ribozyme activity, but inactive ribozyme or antisense controls were not included. Homann and coworkers included antisense and inactive ribozyme controls; they found that their ribozymes inhibited HIV-1 replication in SW480 cells by 90-95%, while the antisense construct and inactive ribozyme inhibited HIV-1 replication by 55-70% (133). In contrast, Lo and colleagues found that antisense transfected Jurkat T-cells were more resistant to HIV-1 replication than cells transfected with an anti-HIV-1 ribozyme (143).

Some of the strongest evidence for engineered ribozyme activity within cells was reported using a hairpin ribozyme targeted to the 5′ untranslated leader sequence of HIV (100). The sequences encoding this ribozyme were placed into a plasmid expression vector, under the control of the human β-actin promoter, and cotransfected into $CD4^+$ HeLa cells with both effector HIV-1 proviral DNA and a CAT reporter plasmid. In their cellular assay, the effector plasmid provided transcripts encoding the HIV-1 *tat* protein, which in turn *trans*-activated CAT expression. The hairpin ribozyme was able to inhibit CAT expression and p24 production up to 80% over control cells. Transfection of HeLa cells with a disabled ribozyme lacking catalytic activity produced about 10% reductions in CAT expression and p24 production, suggesting only a minimal antisense component to the inhibition. Further studies were carried out with the active hairpin ribozyme placed under the control of human $tRNA^{Val}$ or adenovirus VA1 promoters (145). These constructs used in the previously described cotransfection assay showed 10–15% greater inhibition of CAT activity or p24 production relative to the ribozyme transfected from the β-actin promoter. Reductions in HIV-1 mRNA within transfected cells were also directly observed. Further characterization of this ribozyme revealed inhibition of proviral DNA synthesis in the first cycle of infection (146). Finally, several retroviral vectors were constructed containing the active hairpin ribozyme and cotransfected along with DNA constructs from several diverse HIV-1 strains. The active ribozyme was able to inhibit p24 expression from the diverse strains up to 70-95% over control (145).

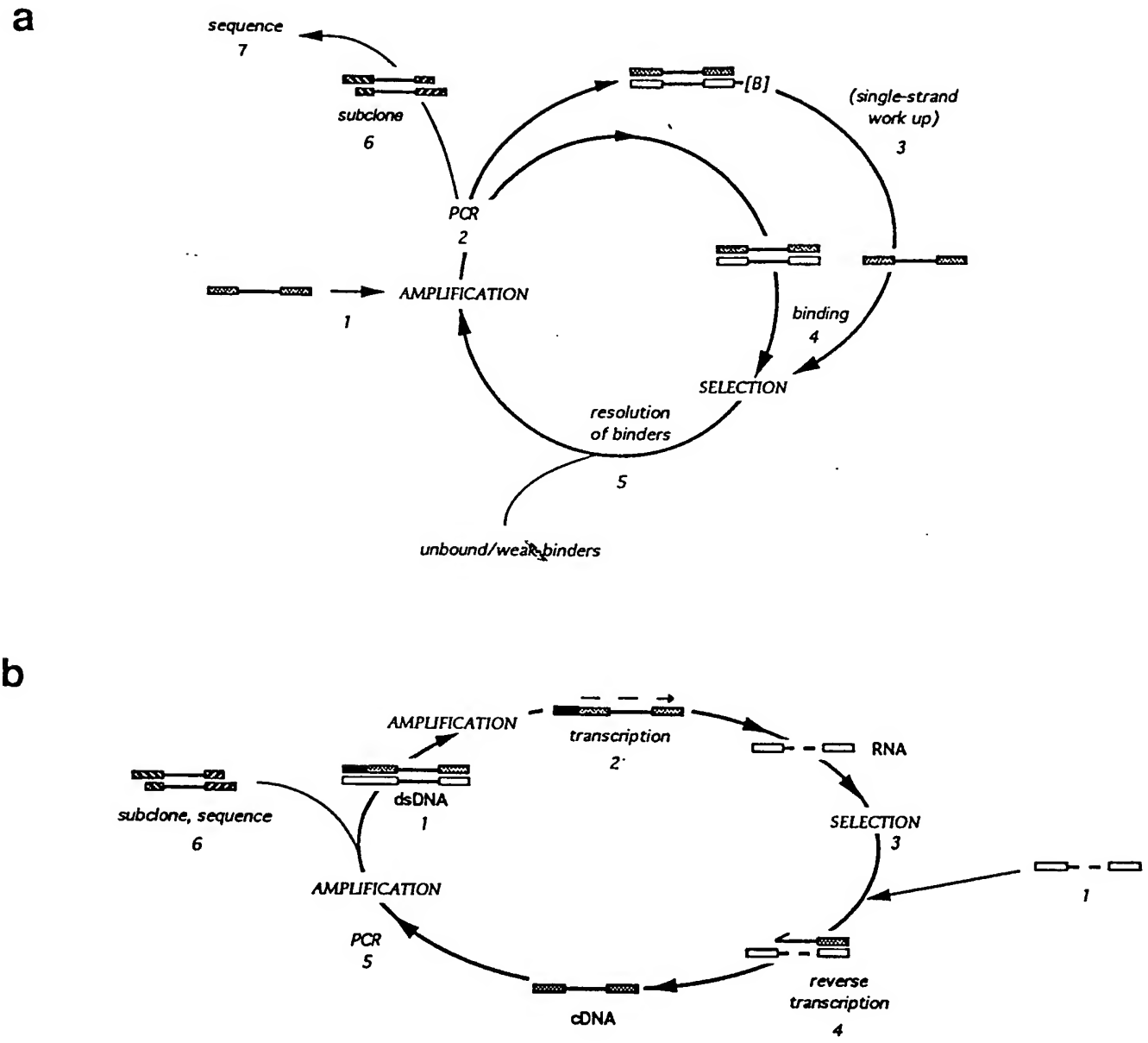

Figure 4. Schematic of *in vitro* selection procedure for single-stranded and double-stranded aptamers (adapted from refs 7, 10). Each cycle of enrichment involves alternating amplification and selection steps. a) *DNA aptamers 1)* A library of oligonucleotide sequences is synthesized in which a randomized region of nucleotides is flanked by two defined PCR primer binding sites (solid gray boxes). *2)* The sequence library is first amplified by large-scale PCR (7) producing double stranded PCR products. *3)* If single-stranded aptamers are desired, the downstream PCR primer is biotinylated at the 5′ end, so that the PCR products can be applied to an avidin-agarose column, followed by elution in weak base to permit recovery of single-stranded DNA oligonucleotides. *4)* The DNA strands are bound to the target protein. The protein may be in solution, or it may be bound to a filter or to the matrix of a chromatography column. *5)* Non-binding sequences are separated from binding sequences by techniques which may include selective elution from a chromatography column (10) or filter (195), or by a gel-electrophoresis band-shift assay (195). The bound DNA sequences are subsequently recovered from the protein, and again subjected to a new round of PCR amplification and binding/selection. After several rounds of enrichment, the final PCR amplification may be performed with modified PCR primers that allow *6)* subcloning into a plasmid, followed by *7)* sequencing of the plasmid. b) *RNA aptamers*. Conceptually, the strategy is similar, although the library of oligonucleotide sequences is amplified by large-scale PCR to produce double stranded PCR products containing a T7 bacteriophage polymerase promoter site (black box). RNA molecules are next produced by *in vitro* transcription using T7 RNA polymerase. The single-stranded RNA pool thus generated is the material allowed to bound the target molecule immobilized on a chromatography column (7). The bound RNA is selectively eluted, reverse transcribed to DNA, and amplified by PCR to produce the second round of double stranded DNA products possessing the T7 RNA polymerase promoter. After several cycles, the PCR step is modified to produce products that allow subcloning into a plasmid restriction site, followed by sequencing of positive clones.

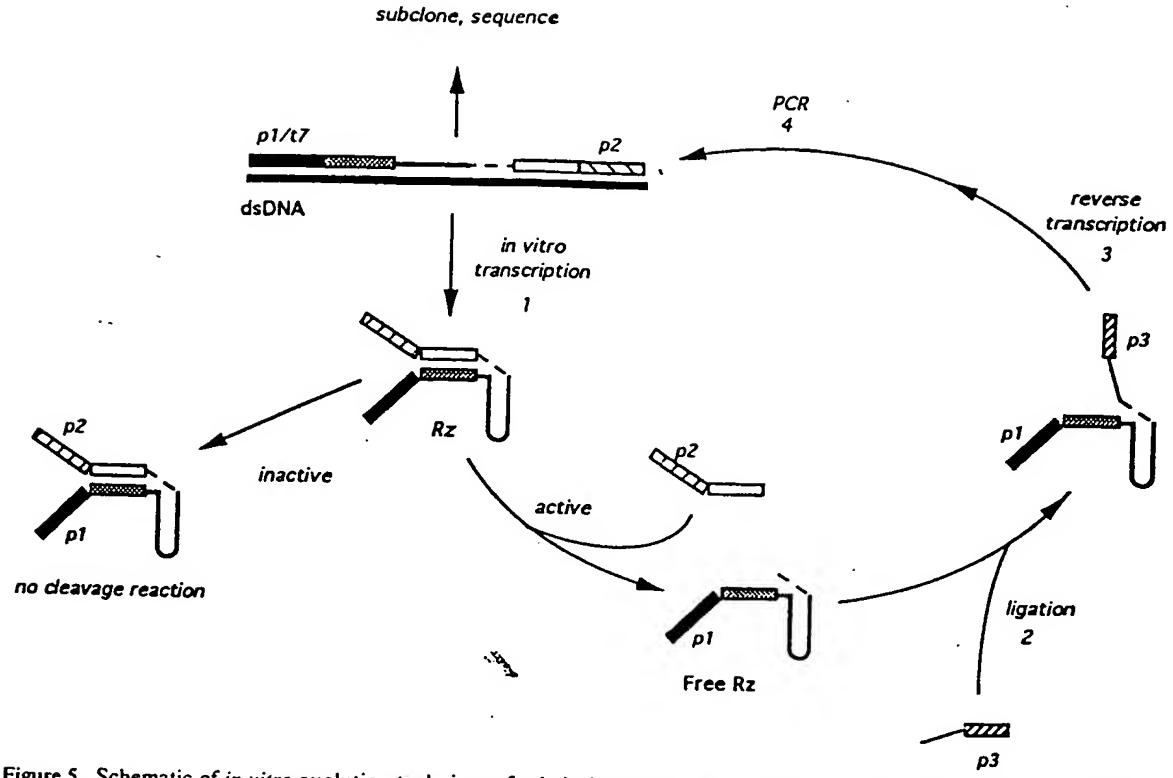

Figure 5. Schematic of *in vitro* evolution techniques for hairpin ribozymes (modified from refs 199, 200). A starting set of mutagenized, sequence degenerate double stranded DNA templates, flanked by defined sequences containing a T7 RNA polymerase promoter (solid black box) adjacent to a defined PCR primer site (*P1*), and a second defined PCR primer site, (*P2*, cross-hatched box), is synthesized. *1) In vitro* transcription of this template pool produces hairpin ribozymes (Rz) in which the substrate sequence (white box) is linked to the ribozyme sequence (gray box and hairpin line) via a polycytosine tether (dashed line). *2)* Active molecules will self-cleave, releasing the substrate and the *P2* priming sequence. *3)* These cleaved molecules, containing a terminal 2',3'-cyclic phosphate, are gel purified and reacted with a large excess of a RNA-DNA oligomer which can ligate to the free ribozyme due to a ribozyme-catalyzed ligation reaction. *4)* The ligation reaction results in introduction of a new priming sequence (*P3*) into the molecule, which serves as an initiation point for cDNA synthesis via reverse transcription. 5) Subsequent selective PCR amplification is performed with primers *P1* and *P3* to return to a double stranded template for further rounds of enrichment. Alternatively, PCR amplification is performed with primers which allow easy subcloning into a plasmid, allowing sequencing of the PCR product. Inactive ribozymes retain the *P2* priming site; thus they may also be reverse transcribed, amplified by PCR, subcloned and sequenced.

Cellular Studies of Ribozymes: Endogenous Substrates. Endogenous genes targeted by *trans*-acting ribozymes include *c-fos* oncogene (130, 131), the *bcr-abl* oncogene (147-150), the H-*ras* oncogene (151, 152), human O^6-methylguanine-DNA methyltransferase (153), the multiple drug resistance gene (175, 213), tumor necrosis factor-α (154), *Drosophila melanogaster fushi tarazu* (*ftz*) gene (134), pleiotrophin (214), β2-microglubulin (216), and the tyrosine kinases *lck* and *fyn* (217). In the studies with the *c-fos* ribozyme, reductions in target RNA and/or protein levels could be attributed to both antisense and ribozyme mediated mechanisms. The studies utilizing the anti-O^6-methylguanine-DNA methyltransferase ribozyme also showed reductions in O^6-methylguanine-DNA methyltransferase activity in one transfected clone, but controls were not included to infer what portion of the inhibition was due to ribozyme versus antisense mediated mechanisms.

The study of the anti-TNF-α ribozyme (154) used preformed ribozymes produced by T7-driven *in vitro* transcription. The ribozymes were transfected using DOTMA (155) into either HL 60 cells or peripheral blood mononuclear (PBMNC) cells. Following the lipofection, the cells were stimulated with PMA and concanavalin A to induce TNF production. Data from both Northern analysis and radioimmunoassay indicated 85-90% reductions in RNA and protein levels in the ribozyme-transfected HL 60 cells, versus 40-60% reductions in cells lipofected with an antisense construct. Similar results were obtained for the PBMNCs.

Each of the studies of anti-*bcr/abl* ribozymes (147-150) showed reductions in *bcl/abl* RNA and p210 protein, and/or reductions in proliferation rates for cells treated with the ribozyme. The study by Shore and coworkers selected individual clones which had been infected by a retrovirus carrying the anti-*bcl/abl* ribozymes. The degree of p210 inhibition varied from clone to clone, and no controls were included to determine the contribution of inhibition by antisense mechanisms (148). Antisense controls were included in the studies by Snyder and colleagues, and Lange and coworkers where it could be concluded that 50-75% of the observed effects were due to antisense mechanisms (147, 148, 150).

Kobayashi and colleagues designed two ribozymes tar-

geted to the multiple drug resistance (*mdr-1*) gene in MOLT-3 cells resistant to trimetrexate (175). *In vitro* studies confirmed ribozyme activity, while cells transfected with the anti-MDR-1 ribozyme showed decreased resistance to vincristine. Cells transfected with an inactive (point mutated) ribozyme showed no changes in vincristine sensitivity. Likewise, a second study of a hammerhead ribozyme targeted to the *mdr-1* gene in pancreatic carcinoma cells (213) demonstrated the presence of the ribozyme decreased both P-glycoprotein expression and resistance to daunorubicin.

Larrson and coworkers constructed several ribozymes targeted toward murine β2-microglobulin (216). These ribozymes were tested in NIH3T3 cells, where up to 80% reductions in β2-microglobulin expression were observed. Transgenic mice bearing the anti-β2-microglobulin ribozyme were also created, in which β2-microglobulin mRNA expression was reduced up to 90% in certain organs of individual mice.

OBSTACLES FACING APPLICATION OF NUCLEIC ACID DRUGS IN CELL CULTURE

Nucleic acid drugs must overcome several formidable obstacles before they can be widely applied as therapeutics. These obstacles require improving the stability of polynucleotide drugs in biological systems, optimizing the affinity and efficacy of the drug without reducing its selectivity, and targeting and delivering nucleic acids across cell membranes.

Stability. As nucleases are ubiquitous in serum and cells, phosphodiester nucleic acids are extremely sensitive to degradation in biological systems. Chemical modifications to nucleic acid polymers have significantly improved the stability of nucleic acid drugs, although these modifications may adversely affect the affinity or activity of the reagent (73, 74; 156-160; reviewed in 6). Stability of nucleic acid drugs may also be improved by introduction of hairpin-forming sequences which stabilize the transcript towards exonucleases (2, 154, 163), provided the hairpin does not adversely affect the affinity of the reagent.

Affinity. The affinity of the nucleic acid drug for its target is a crucial parameter, with implications for both the activity and selectivity of the drug (118). Binding constants on the order of 10-100 nM are likely required for efficient drug activity in cells and *in vivo*. Conjugation of specific intercalating agents to nucleic acids may afford methods for increasing the affinity of the drug without decreasing its specificity (43-45).

The binding of antigene oligonucleotides, engineered ribozymes, and most aptamers, involves specific sequence recognition and hybridization. Sequence selection for aptamers and antigene compounds is fairly restricted. For aptamers, the sequence of the drug is limited by the recognition abilities of the targeted protein; aptamer design may be facilitated by *in vitro* screening and selection techniques (Figure 4); (10, 199). Antigene oligonucleotide sequences are restricted by the duplex target motifs which can sustain triplex formation (Figure 2); hence much effort is being directed at extending triplex formation to regions of double stranded DNA containing all four nucleotides.

Ribozymes must hybridize to RNA targets that possess their own secondary structure, a feature of the target known to affect the efficacy of ribozymes (166). Computer folding programs for RNA may help to determine regions of RNA likely to be involved in RNA structure or help to predict the activity of ribozymes (167, 168). However, these algorithms have not eliminated empirical approaches since the algorithms achieve an 80% accuracy at best, and have not been particularly useful in selecting target sites for antisense inhibition (190, 191). Targeting of the ribozyme by anchor sequences able to hybridize to the RNA at sites distant from the actual cleavage site may provide an alternate strategy for circumventing secondary structure at the ribozyme cleavage site (169).

Maximizing Ribozyme Efficiency. Many engineered *trans*-acting ribozymes do not display catalytic efficiencies near those of natural ribozymes, a problem exemplified in the results of several studies where a 100- to 1000-molar excess of ribozyme over substrate was required for inhibition of target gene expression (90, 135). Improving efficiency and turnover rates of engineered ribozymes is essential if the catalytic potential of these compounds is to be realized. *In vitro* evolution techniques (170, 199) may provide strategies for developing ribozymes which display enhanced catalytic efficiency for particular target nucleic acid molecules (171-174, 207). These strategies apply amplification and selection methods to evolve molecules that are more efficient at a particular catalytic step (Figure 5). Each round of the process provides a new cohort of catalytic RNA that can be improved further (198). However, as mentioned above, the catalytic activity of a ribozyme may be limited by hybridization kinetics and/or release of product which could place an upper limit on the practical efficiency of ribozymes.

Subcellular Distribution. Subcellular distribution of nucleic acid drugs is an important aspect of their activity. Oligonucleotides, when directly introduced into the cytoplasm of living cells, accumulate within the nucleus (192, 193). If the target is in the nucleus, this will be beneficial and the biological properties of antigene oligonucleotides and aptamer compounds will be improved. However, as some mRNAs are localized in the cell (164), RNA targeted reagents (e.g. ribozymes) should colocalize with their substrates if biological effects are to be expected (165).

OBSTACLES TO APPLICATION OF NUCLEIC ACID DRUGS *IN VIVO*

Research with aptamers, antigene oligonucleotides and ribozymes in cell culture has undergone an exponential expansion. The studies listed in Tables II through V indicate these reagents have established themselves as valuable scientific tools in developmental and cell biology. It is clear, however, that application of these expensive compounds *in vivo* requires many problems be solved, some of which have been discussed above in conjunction with studies performed in cell culture, but particularly those related to delivery *in vivo* of nucleic acids to the cytoplasm of specific cells. The delivery challenge can be subdivided into problems with persistence of effect, access to the target cells and efficient cytoplasmic delivery of the drug.

The persistence of effect issue arises because none of the modalities proposed to date can eliminate the disease/target. Thus suppression of disease will require the contin-

ued presence of the agent until the disease is cured or the condition is eliminated. In non-gene therapy approaches, dosing via the intravenous route will be needed at a frequent interval, circa days, if therapeutic levels of the agent are to persist in the body. Although certain sites, such as the eye, may permit less frequent administration of the agent, in most cases, repeated administration via injection will be required. This makes treatment of chronic disorders, such as HIV infection, with synthetic nucleic acid drugs a difficult undertaking.

An obvious solution to the persistence issue for agents that are composed of RNA is to have the patient make their own medication via the gene therapy route (161, 162, 201, 202). This approach reduces the requirement for frequent administration but does not circumvent the other two issues, access and entry into the target cell.

If the target is outside of the vascular system, the agent will have to extravasate. Non-gene nucleic acids drugs have molecular weights in the 3000 to 10,000 dalton range so extravasation is not a particular problem for the agent itself. However, as these drugs do not permeate into the cytoplasm of cells but are found primarily in the endosome compartment (192, 203), they will most likely require some covalent modification or delivery system to mediate their efficient entry into the cytoplasm of the target cell (204). Numerous delivery reagents have been developed to facilitate cellular uptake of oligonucleotides in cell culture (Table VI). These include attempts to modify the ionic backbone, modifications to increase the hydrophobicity (e.g. attachment of cholesterol) as well as attempts to attach a targeting ligand such as biotin or a neoglycoprotein directly to the nucleic acid drug (Table VI). To date these efforts have led to improved uptake but not to improved cytoplasmic delivery. Antibodies have been successfully employed for delivering agents that can amplify their effect, such as toxins, enzymes and free radical generators (206) so they might be useful to deliver a ribozyme but would seem to be less useful for delivery of non-catalytic drugs. Recently a peptide from the Tat protein from HIV has been used to increase protein delivery into cells (230). A molecular conjugate using Tat might enhance the uptake of nucleic acid drugs.

Alternative approaches include the conjugation or association of cationic polymers such as poly-lysine (Table VI) (reviewed in 75) to the nucleic acid drug. Polylysine can increase cell-association and cellular uptake of the nucleic acid drug and has potentiated biological effects for several oligos. Polylysine in an electrostatic complex with oligonucleotides has increased cellular delivery both *in vivo* and *in vitro* (227). The polylysine approach consists of a targeting ligand coupled to the polycation which in turns serves as a molecular glue to attach the ligand to the polyanionic nucleic acid drug. The polylysine approach is a good laboratory tactic to assess various targeting and membrane destabilizing strategies; however it is unlikely to lead to a commercial delivery system because of cytotoxicity and immunologic problems associated with polylysine.

Liposomes (reviewed in 76, 77, 196, 197) or nanoparticles are also being considered for *in vivo* delivery of these agents (204, 205); their advantage is a high payload and the capacity to mediate cytoplasmic delivery. Cationic lipo-

Table VI. Strategies to Enhance Cellular Delivery of Nucleic Acid Drugs

Strategy	Modification	Results	Reference
chemical modifications			
improve ASO uptake by modifying ASO lipophilicity	covalent linkages to cholesterol or thiocholesterol	improved ASO cell association, uptake, nuclear localization; some increase in non-sequence specific effects	219, 220, 221
	covalent linkage to phospholipids	as above	222
	covalent linkage to undecyl or dodecandiol residue	as above	223
improve ASO uptake via conjugation to receptor ligand	covalent linkage to biotin, associate with avidin	improved uptake, but no biological characterization	224
	covalent linkage to neoglycoproteins	improved uptake, but ASO retained in vesicles	225
drug delivery systems			
poly-(L-lysine)	covalent linkage to ASO	enhanced cell association, uptake; potentiation of the biological effects of several antiviral ASOs	75, 226
molecular conjugates with poly-(L-lysine); protein ligands	electrostatic interaction with ASO backbone; targeted via coupled ligand	improved uptake and potentiation of inhibitory effects of ASOs	227
targeted liposomes (immunoliposomes)	encapsulation in multilamellar vesicles coupled to protein A and antibodies	substantial boost in efficacy of ASO; protection from degradation; specificity in cell targeting	228
non-targeted liposomes	encapsulation in liposome, pH-sensitive liposomes	enhanced efficacy of ASO relative to unencapsulated ASOs	229
nanoparticles associated with hydrophobic cations	electrostatic interaction with hydrophobic cations	ASOs protected from degradation, no biological tests reported	205
cationic lipids (COTMA; DOTAP)	electrostatic association between ASO and lipid head group	improved uptake and subcellular distribution	77

somes are effective for *in vitro* delivery of nucleic acid drugs and for *in vivo* gene transfer but to date have not been found useful for delivery of the low molecular weight nucleic acid drugs described in this review. For the microparticulates, vascular cells, cells of the reticuloendothelial system and hepatocytes are potential targets (196, 197). Thus if targeting and entry effectiveness of the microparticulates can be improved then microparticulate systems may be useful for delivery of a variety of nucleic acid drugs currently under development.

In summary advances in molecular biology and synthetic chemistry have led to novel nucleic acid drugs to inhibit gene expression and protein function. However, the delivery and entry of nucleic acid drugs into the target site remains a major obstacle to the successful introduction of this aspect of the molecular biology revolution into a clinical setting.

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